Structural and biochemical characterization of Roco proteins

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Chapter 2

Revisiting the Roco G-protein cycle

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ST and BG performed all biochemical and structural experiments with the bacterial proteins and FH with human LRRK2. AW and AK designed the experiments and all authors contributed to the writing of the paper.

Revisiting the Roco G-protein cycle

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Mutations in leucine-rich-repeat kinase 2 (LRRK2) are the most frequent cause of late-onset Parkinson's disease (PD). LRRK2 belongs to the Roco family of proteins which share a conserved Ras-like G-domain (Roc) and a C-terminal of Roc (COR) domain tandem. The nucleotide state of small G-proteins is strictly controlled by guanine–nucleotide-exchange factors (GEFs) and GTPase-activating proteins (GAPs). Because of contradictory structural and biochemical data, the regulatory mechanism of the LRRK2 Roc G-domain and the RocCOR tandem is still under debate. In the present study, we solved the first nucleotide-bound Roc structure and used LRRK2 and bacterial Roco proteins to characterize the RocCOR function in more detail. Nucleotide binding induces a drastic structural change in the Roc/COR domain interface, a region strongly implicated in patients with an LRRK2 mutation. Our data confirm previous assumptions that the C-terminal subdomain of COR functions as a dimerization device. We show that the dimer formation is independent of nucleotide. The affinity for GDP/GTP is in the micromolar range, the result of which is high dissociation rates in the s^{-1} range. Thus Roco proteins are unlikely to need GEFs to achieve activation. Monomeric LRRK2 and Roco G-domains have a similar low GTPase activity to small G-proteins. We show that GTPase activity in bacterial Roco is stimulated by the nucleotide-dependent dimerization of the G-domain within the complex. We thus propose that the Roco proteins do not require GAPs to stimulate GTP hydrolysis but stimulate each other by one monomer completing the catalytic machinery of the other.

Key words: dimerization, GAD, guanosine-5′-triphosphatase (GTPase), leucine-rich-repeat kinase 2 (LRRK2), Parkinson's disease.

INTRODUCTION

Parkinson's disease (PD) is a common and devastating neurodegenerative disorder affecting 1–2 % of the population over 65 years of age. There is no treatment for PD and the exact cause is unknown [1]. Mutations in human leucine-rich-repeat kinase 2 (LRRK2) are the most frequent cause of late-onset PD [2,3]. They are found in 5–6 % of the patients with familial PD and have also been associated with sporadic PD [4]. LRRK2 belongs to the Roco family of proteins, which are characterized by the presence of leucine-rich-repeats (LRRs) and a tandem of Ras-like G-domain (Roc; Ras of complex proteins), connected to COR (C-terminal of Roc) and often also a kinase domain [5]. The various LRRK2 mutations that have been identified in PD are concentrated in the central region of the protein: mutation on one residue within the LRR domain, one in the Roc domain that can have multiple substitutions, one in the COR domain and two in the kinase domain [6]. The multiple disease-linked mutations in the LRRK2 represent a unique opportunity to biochemically explore the pathogenicity of LRRK2 and identify the therapeutic targets for this neurodegenerative disorder. LRRK2 kinase activity is critically linked to clinical effects and the most prevalent PD mutation, LRRK2 G2019S, in the kinase domain results in an enhanced kinase activity, suggesting a possible PD-related gain of abnormal or toxic function [7–9]. Therefore the function of the LRRK2 kinase domain has been studied extensively and inhibitors of LRRK2 kinase activity are a heavily pursued class of drug targets [10].

The Roc domain of LRRK2 belongs to the family of small G-proteins [5]. G-proteins are GTP-binding proteins which switch between an active GTP- and an inactive GDP-bound state. In Roco family members, the G-domain always occurs in tandem with the COR domain. Contradictory biochemical and structural data have been reported for the LRRK2 RocCOR module, therefore its function is still not well understood [11–13]. However, it is known that the G-domain of LRRK2 functions as a *bona fide* GTP-binding protein and that GTP binding is essential for the regulation of LRRK2 kinase activity [14–18]. Our previous studies with the Roco protein from *Chlorobium tepidum* suggested that COR is a dimerization device and that stimulation of the GTPase activity depends on dimerization [12]. However, the structure of the RocCOR domain from *C. tepidum* was solved in the absence of nucleotide. Therefore the effect of nucleotide binding on the structure, in particular its effect on the switch regions of the protein, needs to be investigated. We also need to show and/or confirm whether COR dimerization, the low nucleotide affinity and low GTPase activity is a general feature of the Roco proteins and qualifies them as G-proteins activated by dimerization (GADs). In the present study, we investigated the structure and the function of the RocCOR module in more detail. Our results show that the Roco proteins have a relatively low affinity for and fast dissociation of nucleotide, COR functions

Abbreviations: COR, C-terminal of Roc; GAD, G-protein activated by dimerization; GAP, GTPase-activating protein; GEF, guanine-nucleotide-exchange factor; GppNHp, 5′-guanylyl imidodiphosphate; HEK, human embryonic kidney; HRP, horseradish peroxidase; LRR, leucine-rich-repeat; LRRK2, leucine-rich-repeat kinase 2; mart, 30-*O*-(*N*-methyl)anthranoyl; PD, Parkinson's disease; Roc, Ras of complex proteins; SEC, size-exclusion chromatography; TEV, tobacco etch virus.

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as a dimerization device and dimerization is essential for GTPase activity.

EXPERIMENTAL

Recombinant expression and purification of Roco proteins

The coding sequence of the *Methanosarcina barkeri* Roco2 (Swiss: Q46A62) Roc (amino acids 318–480) and RocCORΔC (amino acids 287 –629) fragments were cloned into a pGEX4T1 vector containing an N-terminal GST tag and TEV (tobacco etch virus) cleavage site. The RocCOR (amino acids 287–790) fragment was ligated into pProExHTb, which contains an N-terminal histidine tag. The RocCOR R404A/R405A mutant was created by the Quick Change method and verified by sequencing. The resulting expression plasmids were transformed in BL21(DE3) cells and grown in Terrific broth (TB) medium. Expression of the GST–Roc, His–RocCOR and GST–RocCORΔC fragments was induced with 0.1 mM IPTG at a D_{600} of 0.7 in shaking culture. Utilizing the LEX bioreactor (Harbinger Biotech), the cells were grown to a D_{600} of 3 before induction. After overnight expression at 18 °C, cells were harvested by centrifugation, washed (resuspended and pelleted) with TBS and resuspended in buffer A (30 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 3 mM 2-mercaptoethanol, 0.1 mM GDP and 5 % glycerol). Cells were lysed using sonication and purified by GSH or Ni–NTA (nitrilotriacetic acid)/Talon affinity chromatography. The protein was eluted in buffer A containing either 20 mM GSH or 200 mM imidazole. GST-fusion proteins were cleaved with TEV protease. Subsequently size-exclusion chromatography (SEC) with buffer A was performed.

Size determination by SEC

Before performing the SEC experiments, all proteins were loaded with GppNHp (5′-guanylyl imidodiphosphate), GDP or GDP with 2 mM $AlCl_3$ and 20 mM NaF (GDP + AlF_x) by incubation with 10 mM EDTA and a 20-fold excess of the indicated nucleotides for 2 h at room temperature. SEC experiments were performed on S75 (10/300) and S200 (10/300) superdex columns (GE Healthcare). To keep the protein in a nucleotide-bound state during the experiment, an excess of the corresponding nucleotide (0.1 mM GppNHp, 0.1 mM GDP, 0.1 mM GDP + 20 mM NaF + 2 mM $AlCl_3$) was added to the SEC buffer (30 mM Tris/HCl, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 3 mM 2-mercaptoethanol and 2.5 % glycerol).

Cell culture and immunoprecipitation

Human embryonic kidney (HEK)-293T-cells were maintained in Dulbecco's modified essential medium supplemented with 10 % FBS and incubated in 37 °C/10 % CO_2 incubator. Transfection was performed by standard calcium phosphate method [19]. After transfection with appropriate plasmids for 48 h, cells were lysed in lysis buffer containing 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 100 μM GDP, 2 mM DTT, 5 % glycerol and protease inhibitors (10 μg/ml leupeptin, pepstatin A, aprotinin, 1 mM benzamidine and 1 mM PMSF). Immunoprecipitation was performed by mouse anti-FLAG (M5) antibody (Sigma) or mouse anti-GFP antibody (Roche) with Protein A/Protein G-Plus agarose (Merck) overnight at 4 °C. The precipitated samples were washed four times with lysis buffer and resuspended in 1X Laemmli buffer. Samples were separated by SDS/PAGE and transferred on to PVDF membrane (Whatman). Rabbit anti-FLAG (M2) antibody (Cell Signaling), mouse anti-GFP (Roche) or rabbit anti-GFP (Cell Signaling) were used for primary antibodies. Rabbit anti-mouse HRP (horseradish peroxidase)-conjugated antibody and HRP-conjugated Protein A were used as secondary antibodies for mouse and rabbit primary antibodies. Blots were incubated with SuperSignal West Pico chemiluminescent substrate (Pierce) for signal development and detected by exposure to X-ray film (Fuji).

Crystallography

The RocCORΔC protein crystallized in 50 mM Tris/HCl (pH 8.5), 50 mM Na_2SO_4, 50 mM Li_2SO_4 and 30 % (w/v) PEG400 in hanging drop. For cryoprotection, another 20 % glycerol was added to the reservoir solution. The dataset was collected on beamline X10SA at the Swiss Light Source (Paul Scherrer Institute, Villigen, Switzerland) and was indexed, integrated and scaled with the XDS package. The structure was solved by molecular replacement using the Roc domain of *C. tepidum* (PDB code 3DPU) as a template. The model was built in COOT and refined with REFMAC5 (CCP4 suite). Figures were generated in PyMOL (DeLano Scientific).

GTPase activity

To measure the GTPase activity, 5 μM *M. barkeri* RocCORΔC, RocCOR or RocCOR R404A/R405A Roco protein was incubated in buffer A (without GDP) with 200 μM GTP in the presence of [γ-^{32}P]GTP. Samples were taken at the indicated time-points and immediately quenched by mixing with ice-cold 20 mM phosphoric acid with 5 % activated charcoal. All non-hydrolysed GTP was stripped by charcoal and precipitated by centrifugation. The inorganic phosphate remaining in the supernatant was subjected to scintillation counting.

Nucleotide affinity

To remove the bound nucleotide, the *M. barkeri* RocCOR fragment was incubated overnight at 4 °C with alkaline phosphatase (1.5 units/mg protein). HPLC was used to monitor degradation of the GDP. Subsequently, SEC was performed with buffer A (without nucleotide) to obtain nucleotide-free protein. The affinity for GDP and GppNHp was measured employing 30-*O*-(*N*-methyl)anthranoyl (mant)-nucleotides (1 μM). The protein was titrated stepwise at 20 °C until saturation was reached. Fluorescence-polarization was measured using the FluoroMax-4 (Horiba Scientific). Mant-nucleotides were excited at 366 nm and emission was recorded at 450 nm. The signal was integrated over at least 5 min. The dissociation constant (K_D) was calculated by fitting a quadratic equation to the data using GraFit5 (Erithacus Software).

RESULTS AND DISCUSSION

Dimerization is independent of nucleotide binding

Because of the lack of sufficient amounts of stable recombinant human Roco protein and to support and extend previous data from a bacterial homologue [12], we used *M. barkeri* Roco proteins to study the dimerization mechanism in more detail. Previously we have shown that homologous Roco-proteins from bacteria and *Dictyostelium* can be used to infer/deduce the biochemical and structural properties of LRRK2 [12,20]. *M. barkeri* possesses four

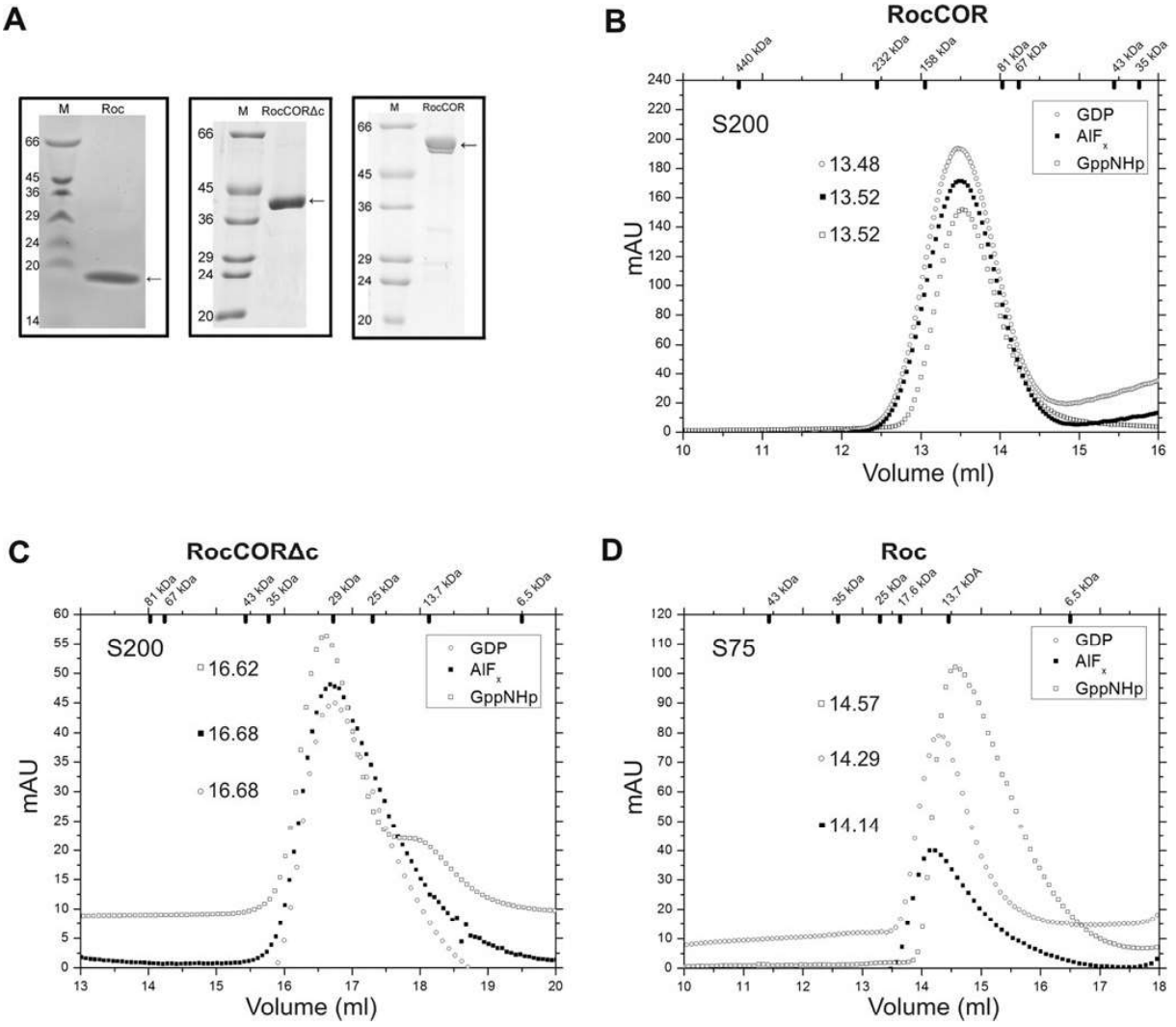


Figure 1 SEC experiments of *M. barkeri* RocCOR

(**A**) Coomassie Blue-stained SDS/PAGE gels showing the final purity of the purified *M. barkeri* Roco2 fragments. (**B–D**) SEC analysis of (**B**) RocCOR (amino acids 287–790), (**C**) RocCORΔC (amino acids 287–629) and (**D**) Roc (amino acids 318–480) in the presence or absence of excess of the indicated nucleotides. Apparent molecular masses were obtained from equilibration with standard marker proteins (GE Healthcare). AU, arbitrary units.

Roco proteins; all consisting of LRRs, a Roc- and a COR domain. In contrast with the human LRRK2 protein, we were able to express and purify a Roc (amino acids 318–480), a RocCORΔC (amino acids 287–629) and a RocCOR (amino acids 287–790) fragment from *M. barkeri* Roco2 in *Escherichia coli* (Figure 1A and Supplementary Figure S1). RocCORΔC does not include the C-terminal subdomain of COR, which we have previously shown to be responsible for dimer formation of RocCOR from *C. tepidum* [12]. By SEC we analysed the oligomeric state of the different Roco constructs. The RocCOR construct elutes at an apparent mass of 122 kDa, indicating that it forms a dimer in solution (Figure 1B). In contrast, the Roc and the RocCORΔC constructs are both monomeric in solution, with an apparent mass of 12 kDa and 39 kDa (Figures 1C and 1D), showing that the C-terminal subdomain of *M. barkeri* COR is essential for dimerization.

To test if the dimerization of the *M. barkeri* RocCOR tandem is nucleotide dependent, we loaded the protein with GDP (inactive), GppNHp (active) or GDP + AlF$_x$ (mimic of the transition state during GTP hydrolysis) and subsequently performed SEC experiments (Figures 1B–1D). To ensure that the protein was nucleotide-bound during the complete course of the experiment, an excess of the nucleotides was added to the buffer. The size exclusion experiments revealed only very small nucleotide-dependent shifts in the apparent masses of the three constructs, probably reflecting nucleotide-dependent structural changes (see also below), suggesting that dimer formation is constitutive and not regulated by the nature of nucleotide bound to the Roc domain.

Table 1 Data collection and X-ray refinement statistics

Values in parentheses are for the highest resolution shell. 1 Å = 0.1 nm

Crystal	RocCOR∆C
Data collection	Native
Space group	$P6_122$
Cell dimensions	
a, *b*, *c* (Å)	109.4, 109.4, 223
α, β, γ (°)	90, 90, 120
Resolution (Å)	49 (2.9)
R-measured	0.073 (0.8)
I/σ	21.84 (2.84)
Completeness	99.6 (98.6)
Redundancy	8 (8)
Refinement	
Resolution (Å)	2.9
Total number of reflections	17273
Number of reflections used	16363
R_{work}/R_{free}	0.24/0.27
Number of atoms	
Protein	2643
Ligand/ion	48
Water	30
B-factors	
Protein	62.31
Ligand/ion	78.12
Water	70.39
RMSD	
Bond lengths (Å)	0.005
Bond angles (°)	0.985

The structure of GDP-bound monomeric Roc

Since the previous structure of the Roc domain from *C. tepidum* was solved in the absence of nucleotide, we set out to analyse nucleotide binding to the Roco G-domain at the molecular level. We were able to crystallize and solve the structure of GDP-bound *M. barkeri* RocCOR∆C (Figure 2). Crystals were obtained in space group P6(1) 2 2 and the structure was solved by molecular replacement using the Roc domain of *C. tepidum* Roco1 (PDB code 3DPU) as a template (Table 1). A single RocCOR∆C molecule was found in the asymmetric unit. The Roc domain shows the typical canonical monomeric G-protein fold with a central six-stranded β-sheet and α-helices on each side of the sheet (Figures 2A and 2B). Helices, β-sheets and the highly conserved G1–G5 motifs are arranged as reported for other monomeric G-proteins (Figure 2B) [21]. The G1 motif, also called the P-loop, is essential for the binding of the β-phosphate of the nucleotide, as well as for the interaction with a magnesium-ion in the nucleotide-binding pocket. The G1 motif loop of RocCOR∆C (GDGEAGKT) and the guanine-base-binding G4 (NKID) and G5 (normally SAK, here SCK) motifs overlay notably with the Ras structure and the magnesium-ion is coordinated with the β-phosphate in the canonical fashion. The switch regions, which are the sensors of the nucleotide (GDP/GTP) state of G-proteins and required for interaction with effectors, have a particular conformation different from that of other G-domains, as expected.

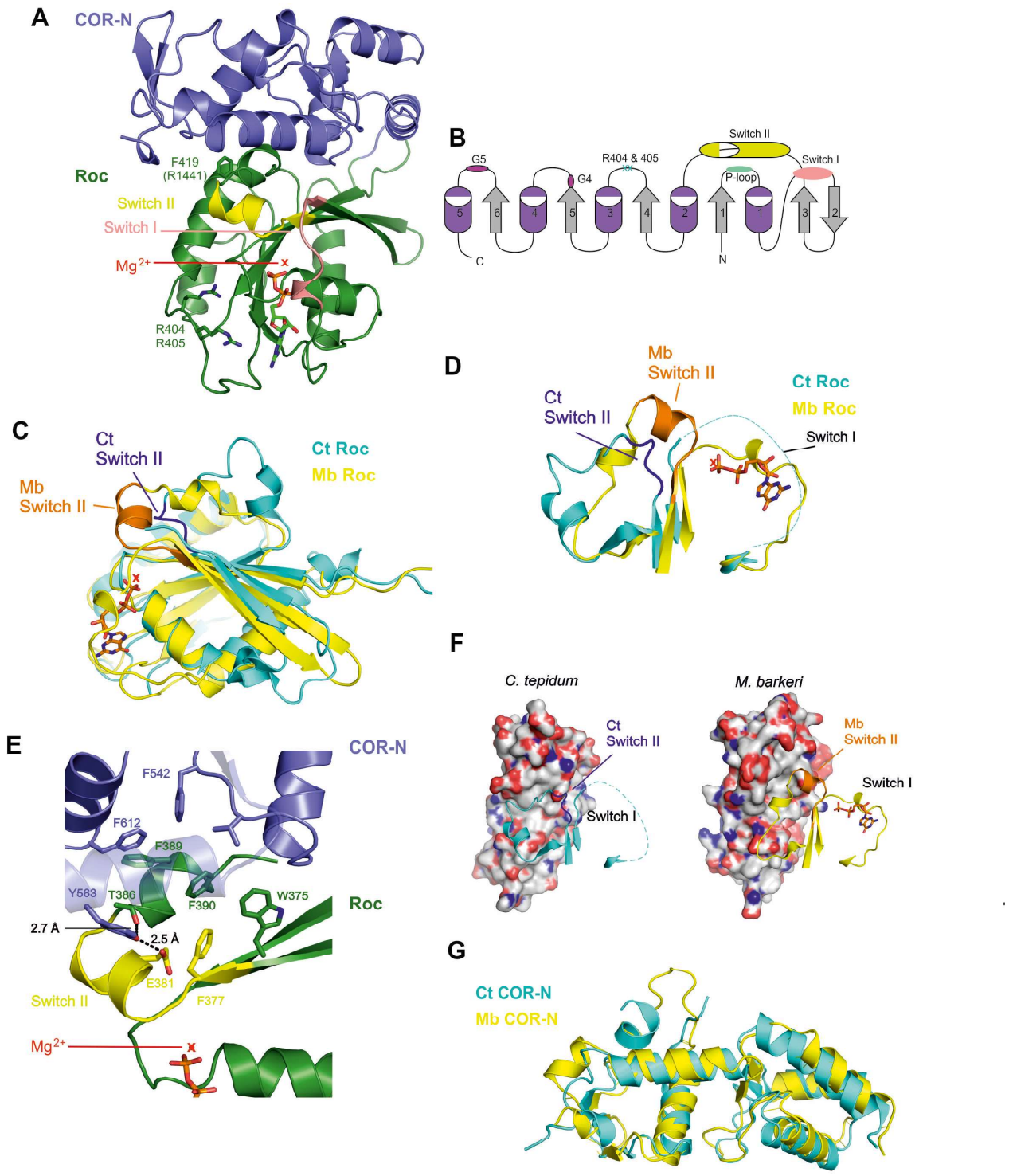
Superimposition of the swapped dimer structure from the human Roc dimer (PDB code 2ZEJ) [12] with the Roc domain from *C. tepidum* had revealed serious clashes between the second G-domain and the N-terminal subdomain of COR [12]. Importantly, the latter region is highly conserved in all Roco proteins, including LRRK2 (Supplementary Figure S1) [5]. The overall fold of the N-terminal part of COR (COR-N) in the monomeric RocCOR∆C structure is highly similar to that in the previously described *C. tepidum* RocCOR dimer (Figure 2C) [12]. Although the RocCOR∆C COR-N is slightly tilted, the interactions between Roc and COR-N are highly similar in the *M. barkeri* and *C. tepidum* structures.

Although switch 1 could not be traced in the *C. tepidum* structure, it can be clearly identified in the Roc COR∆C structure. Whereas switch 2 comprises a loop between β-strand-3 and helix-2 in most Ras-like proteins [21], in RocCOR∆C, switch 2 consists of a short helix. Helix α2 is moved towards the nucleotide-binding pocket in order to allow the switch 2 loop to form this additional helix (Figures 2C and 2D). Whereas switch 1 is oriented towards the solvent, switch 2 is situated in the conserved Roc/COR interface (Figure 2E). There are a number of hydrophobic side chains from helix-2 and switch 2 pointing into the hydrophobic pocket between Roc and COR and an additional hydrogen bond interaction between Tyr^{563} from COR and Glu^{381} and Thr^{386} in switch 2 of Roc (Figure 2E). In comparison with the nucleotide-free *C. tepidum* Roc domain, the present GDP-bound Roc domain structure reveals a major rearrangement of switch 2 which influences the orientation of COR-N (Figures 2D and 2F). In the *M. barkeri* structure, the COR-N is shifted with respect to the Roc domain. Neglecting this relative shift in orientation, the G-domain (Figure 2C) and COR-N (Figure 2G) superimpose rather well.

The main function of the small and large G-proteins is to structurally switch between a GDP- and a GTP-bound state and to thus allow coupling to a downstream effector. Assuming that the Roc domain acts as a structural switch and there is no indication that it would not, we might assume from structural considerations that the nucleotide state mediates intramolecular conformational changes between the domains. Switch 2 is a very promising candidate to be a mediator of such intramolecular signalling through the Roc/COR domain interface. In such a scenario, the GDP–GTP transition would cause a conformational change in switch 2 and in the Roc/COR interface with implications for the activation mechanism of the protein. A strong argument for such a mechanism comes from the fact that the Roc and COR always occur in tandem and that the interface residues are highly conserved from bacterial to human Roco proteins (Supplementary Figure S1). The significance of the interface is highlighted by the finding that patients with LRRK2 mutations of the Roc and COR domains are most probably situated in this interface [12].

Dimerization is essential for GTPase activity

To measure the GTPase activity, the recombinant *M. barkeri* proteins were incubated with [γ-^{32}P]GTP and the subsequent P_i release was measured over time (Figure 3). The RocCOR dimer showed a hydrolysis activity of 1.3×10^{-2} min^{-1} (Figure 3), which is in a similar range as reported for the *C. tepidum* and LRRK2 Roco proteins [12,13,22] and similar to what has been reported for Ras. Previously, it was shown that GTPase activity of *C. tepidum* is regulated by dimerization [12]. Consistent with this, the RocCOR∆C monomer (1.7×10^{-3} min^{-1}) shows an approximately 10-fold decreased GTPase activity compared with the RocCOR dimer (Figure 3). *C. tepidum* Roco uses an arginine finger that is essential for stimulating GTP hydrolysis in the neighbouring Roc domain [12]. A detailed look at the *M. barkeri* RocCOR∆C structure revealed two arginine residues (Arg^{404} and Arg^{405}) that are located in the same loop and in a position to point towards the γ-phosphate, similar to the *C. tepidum* Roco arginine finger (Arg^{543}) (Figure 2A; Supplementary Figure S1).



(**A**) Ribbon diagram of the RocCOR△C fragment (287–629). The Roc domain (green) is bound to GDP (ball and stick) and contains β-phosphate-bound magnesium (red cross). Structural elements of a canonical G-protein such as switch 1 and 2 are highlighted. The COR domain is shown in blue. (**B**) Topology diagram of the *M. barkeri* Roc domain. Structural elements of a canonical G-protein are highlighted corresponding to (**A**). (**C**) Overlay of *C. tepidum* (cyan) and *M. barkeri* (yellow) Roc domains. The switch 2 regions are highlighted in blue and orange respectively. (**D**) Close up of the switch regions highlighting the rearrangement of switch 2 when nucleotide is bound. The dotted line represents switch 1 of *C. tepidum* Roc domain which was not resolved in the structure. (**E**) The hydrophobic interface between the Roc domain (green) and the COR domain (blue) in close proximity to the switch 2 region (yellow). The hydrogen bonds between Thr^{386}, Tyr^{563} and Glu^{381} are shown in black. Hydrophobic residues forming the interface are highlighted (ball and stick). (**F**) Roc/COR interface shown by the surface representation of the N-terminal subdomains of COR (COR-N) from *C. tepidum* and *M. barkeri* with switch regions of the Roc domain in ribbon representation. Switch 2 lies within the RocCOR interface The rearrangement of switch 2 also influences the orientation of COR-N. (**G**) Overlay ribbon diagrams of COR-N of *M. barkeri* (yellow) and *C. tepidum* (cyan).

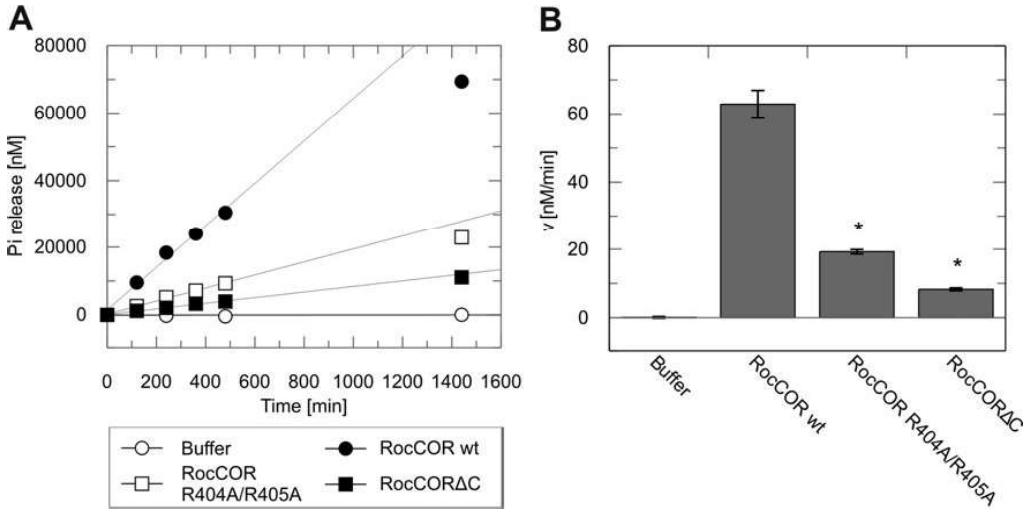


Figure 3 GTPase activity

(**A**) GTP hydrolysis rates of *M. barkeri* wt (wild-type) RocCOR, RocCOR(R404A/R405A) and RocCORΔC as measured by multiple turnover charcoal assays, with 5 μM protein and 200 μM [γ-^{32}P]GTP, as described in the Experimental section. The experiment was repeated at least two times. (**B**) Initial rates in nmol/min of the GTPase experiments shown in (**A**). *$P < 0.01$ compared with wt RocCOR.

The dimeric RocCOR R404A/R405A double mutant showed a similarly low GTP hydrolysis rate (3.9×10^{-3} min^{-1}) as the RocCORΔC monomer (Figure 3). This strongly suggests, by analogy to the mechanism of the *C. tepidum* protein, that within the RocCOR dimer, the arginine finger of one monomer completes the catalytic machinery of the other monomer.

Roco proteins have low nucleotide affinity

Classical G-proteins have a very high affinity for nucleotides in the nanomolar to picomolar range and therefore need GEFs (guanine-nucleotide–exchange factors) to accelerate nucleotide dissociation and thus enable the exchange from GDP to the more abundant GTP [23]. Titration of the nucleotide-free *M. barkeri* RocCOR protein with fluorescent-labelled mant-GDP or the GTP analogue mant-GppNHp revealed that binding of nucleotide can be saturated with an equilibrium constant of 1.8 μM and 8.1 μM respectively (Figure 4A). Highly similar nucleotide affinities were reported for the Roc domain of human LRRK2, *Dictyostelium* GbpC and *C. tepidum* [12,13,15], suggesting that all Roco proteins have a relatively low nucleotide affinity (micromolar range). Correspondingly, using mant-GDP and stop flow analysis, we found GDP dissociation rates of 0.33 s^{-1} and 0.17 s^{-1} for RocCOR and Roc respectively (Figure 4B). This shows that the nucleotide dissociation from Roc is not influenced by COR and that GEFs are not required for activation in a physiologically relevant time frame.

LRRK2 dimerizes via the COR domain

It has been shown that LRRK2 also forms a dimer with low nucleotide affinity and slow GTPase activity and that dimerization is essential for kinase activity [13,22,24,25]. Although the biochemical data reported for the bacterial proteins and LRRK2 complement each other, the 3D structure of *C. tepidum* RocCOR and the *M. barkeri* RocCORΔC structure shown in the present paper contradict the structure of the LRRK2 Roc domain in that dimerization via COR would produce a serious clash in LRRK2. Thus the dimerization mechanism of LRRK2 is still unclear [11,12]. Whereas the Roc domain of *C. tepidum* and *M. barkeri* have a normal G-domain fold, the LRRK2 Roc domain was found as domain-swapped, in which the N-terminal part of one G-domain interacts with the C-terminal of the other, forming a constitutive dimer [11]. However, Deng et al. [11] could not convincingly show dimer formation of the Roc domain in solution, whereas Liao et al. [13] showed that the human LRRK2 Roc domain forms primarily a monomer in solution.

In the present study, we performed co-immunoprecipitation experiments in lysate of HEK-293T-cells (Figure 5). Various GFP-fused truncated LRRK2 constructs were co-expressed with FLAG-tagged LRRK2, immunoprecipitated and the pull-down fraction was visualized by Western blotting (Figures 5A and 5B). FLAG-tagged RocCOR (amino acids 1293–1840) strongly interacts with GFP-fused RocCOR and a similar strong interaction was detected with the COR (amino acids 1511–1840) domain alone (Figure 5A, lanes 4 and 2). In contrast, GFP–Roc (1334–1516) is not co-purified with FLAG-tagged RocCOR (Figure 5A, lane 1), supporting the notion that COR is necessary for dimerization. When using GFP–RocCORΔC (amino acids 1293–1674), which lacks the C-terminal part of the COR domain (CORΔC), less FLAG-tagged RocCOR was co-precipitated (Figure 5A, lane 3) when compared with using GFP–RocCOR as a bait (Figure 5A, lane 4). Furthermore, FLAG- or GFP-tagged COR only showed strong interactions with constructs that contain the complete COR domain (Figure 5B, lanes 4–6), whereas Roc alone is unable to interact with Roc, COR or RocCORΔC (Figure 5B, lanes 1, 2 and 4). RocCORΔC cannot be co-purified with Roc, COR or RocCORΔC (Figure 5B, lanes 2, 3 and 5), further supporting the role of the C-terminal subdomain.

Together these experiments show that the bacterial RocCOR tandem is a good mimic of the human RocCOR tandem from LRRK2 and that the C-terminal subdomain of LRRK2 COR is essential for stable dimer formation (see above) [12].

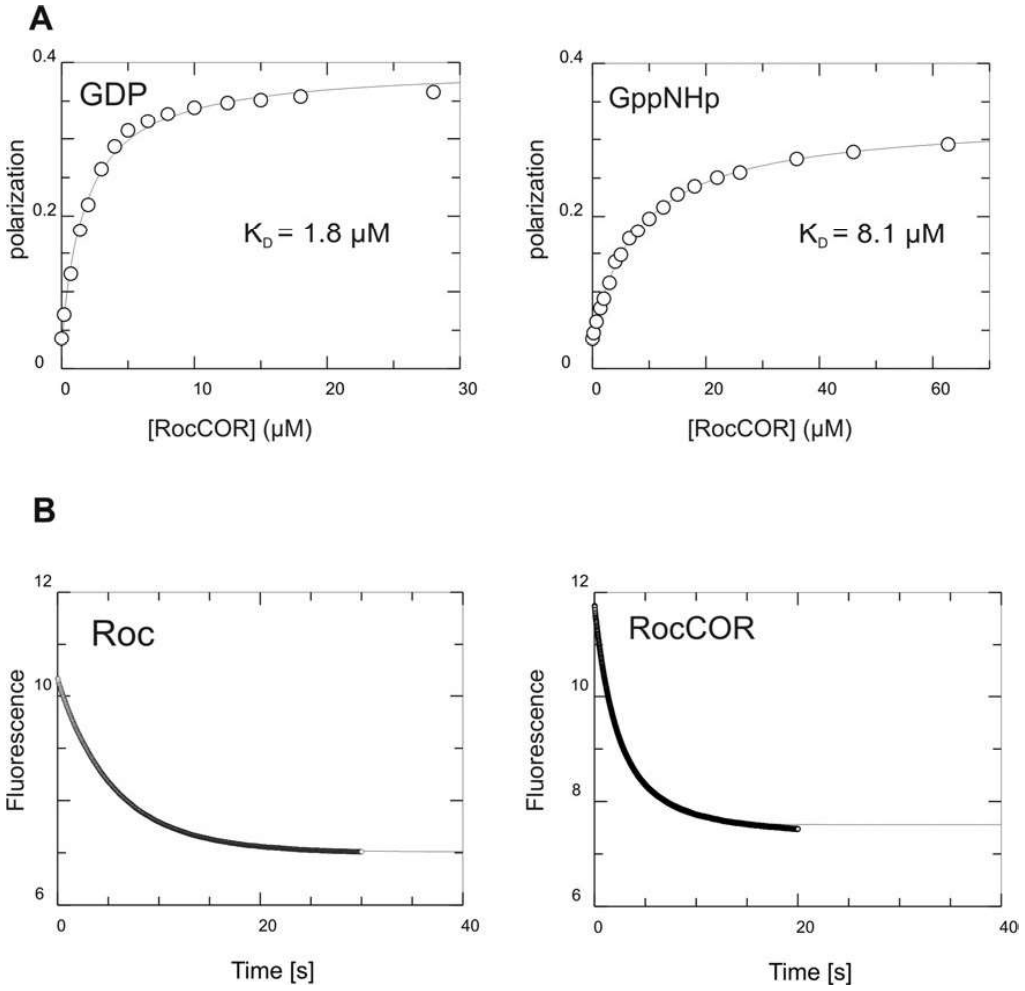


Figure 4 Roco proteins have a low nucleotide affinity

(**A**) Affinities of mant-nucleotides to *M. barkeri* RocCOR measured by fluorescence-polarization. The protein was added stepwise to 1 μM mant-GDP (left) or mant-GppNHp (right). The experiment was repeated three times and for calculating the equilibrium constant; the data were fitted to a quadratic equation. (**B**) Stopped-flow measurement of GDP dissociation from *M. barkeri* Roc and RocCOR. mant-GDP (1 μM) was pre-incubated with 10 μM of protein in GDP-free buffer A (see the Experimental section). GDP (200 μM) was injected and the fluorescent change was monitored over time (excitation at 366 nm, emission at 450 nm, 20°C). The experiment was repeated five times and values were averaged. The resulting curves were fitted with a single exponential curve to calculate the dissociation rate constant k_{off} using GraFit (Erithacus Software).

Functional cycle of Roco proteins

The cycle of 'classical' small G-protein is strictly controlled by GEFs, which catalyse the exchange from GDP to GTP, whereas the intrinsically-low GTP hydrolysis is increased by GAPs (GTPase-activating proteins) [21]. There are a few reports suggesting GAPs and GEFs for LRRK2, however, none of them could show binding of these putative regulators to the Roc domain or any significant increase in the corresponding rates [26–28]. All the Roco proteins studied thus far, including LRRK2, show a much lower nucleotide affinity when compared with 'classical' small G-proteins and therefore most probably do not need GEFs for activation [12,15]. It is well established that LRRK2 and other Roco proteins are active as dimers [12,29,30]. We thus interpret our data to show that Roco proteins, including LRRK2, belong to the GAD family of G-proteins [31]. Roco proteins are dimers, most probably constitutive, by interaction via the COR domains. Based on the available Roco structures and by analogy to other GAD proteins [31], we speculate that in the GDP-bound inactive state the G-domains are flexible, but in the active form the G-domains come into close proximity to each other. This GDP–GTP transition is most probably causing conformational change in other parts of the protein and subsequently in its activation. However, to completely understand this step of the activation mechanism it will be essential to determine how the different Roco/LRRK2 domains interact with each other to create the biological output of the protein. The GTPase reaction is also dependent on dimerization, because efficient catalytic machinery is formed by complementation of the active site of one protomer with that of the other protomer. In this way, the GTPase reaction functions as a timing device for the biological function of the protein.

Two common PD-related mutations have been found in the RocCOR domain: Arg^{1441} with multiple substitutions

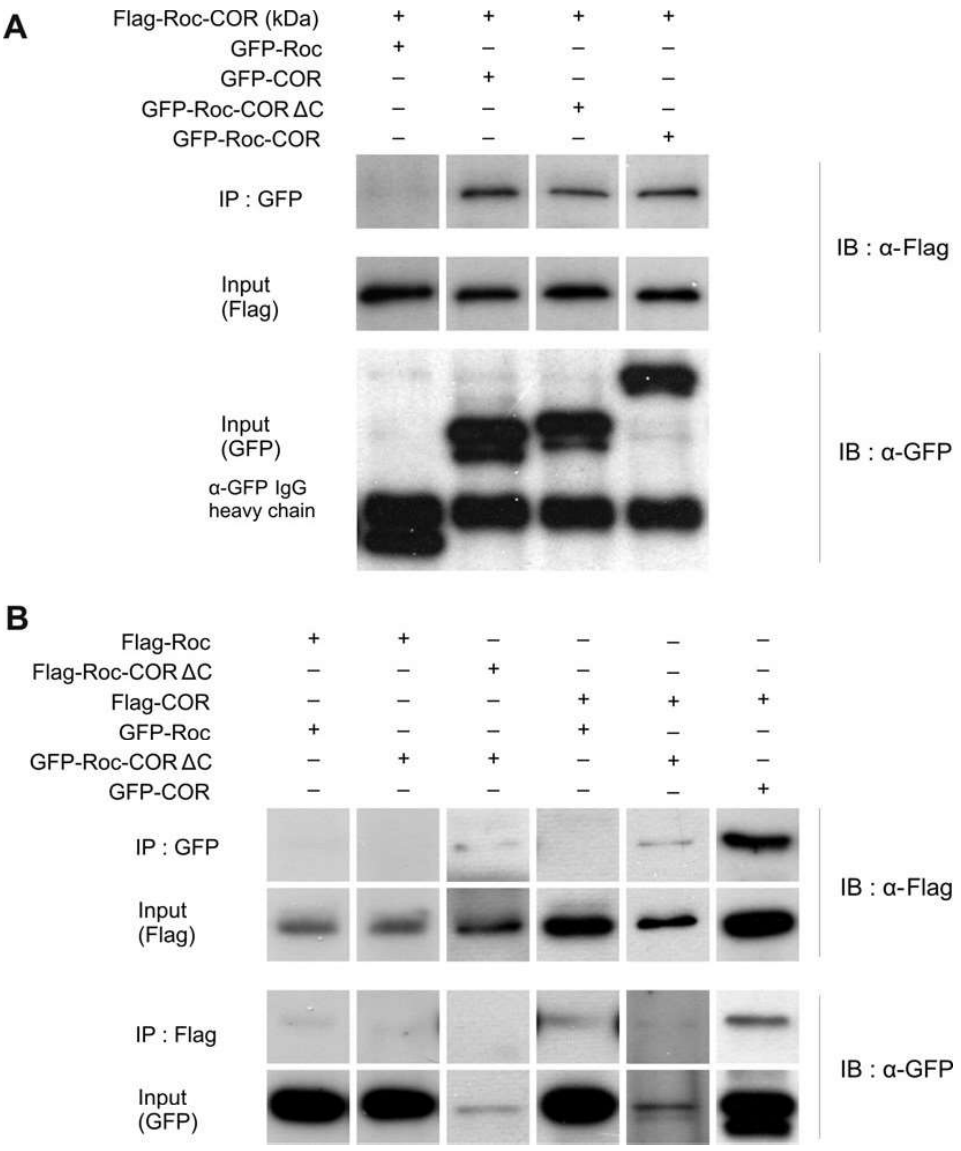


Figure 5 Immunoprecipitation of human LRRK2 domain fragments

(**A**) FLAG-tagged RocCOR was co-expressed with GFP-tagged Roc, COR, RocCOR ΔC and RocCOR in HEK-293T-cells. Anti-FLAG (M5) antibody (Sigma) and anti-GFP antibody (Roche) were used for precipitating the FLAG- and GFP-tagged proteins respectively. Immunoprecipitated samples were separated by SDS/PAGE and subjected to Western blotting with anti-FLAG (M2) antibody (Cell Signaling) or anti-GFP antibody (Roche). (**B**) The indicated LRRK domains tagged with either FLAG or GFP were co-expressed in HEK-293T cells and immunoprecipitated as described for (**A**).

(cysteine/glycine/histidine) in the Roc domain and Y1699C in the COR domain [2,3]. Previous studies showed that both the PD mutations in the Roc and the COR result in decreased GTPase activity [14,16,17]. LRRK2 (Arg^{1441}) is not conserved in prokaryotes; the corresponding residue in *M. barkeri* Roco would be Phe^{420} (Supplementary Figure S1). Consistent with the previous data, this residue points into the hydrophobic interface between the Roc and the COR (Figure 2A) [12]. Furthermore, the *C. tepidum* structure showed that the conserved LRRK2 COR Tyr^{1699} residue also points into the same intradomain interface [12]. This strongly suggests that the reduced GTPase activity of these LRRK2 PD-mutants is most probably caused by altered interactions between the Roc and COR domains in the dimer. Importantly, this also implies that targeting the RocCOR domain may be an interesting therapeutic approach. Therefore it will be important to get a more detailed understanding of the complete G-protein cycle of LRRK2. Structures of Roco proteins in the different nucleotide states will be important in this enterprise.

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AUTHOR CONTRIBUTION

Susanne Terheyden and Bernd Gilsbach performed all biochemical and structural experiments with the bacterial proteins and Franz Ho with human LRRK2. Alfred Wittinghofer and Arjan Kortholt designed the experiments and all authors contributed to writing of the paper.

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Supplementary Information

Figure S1

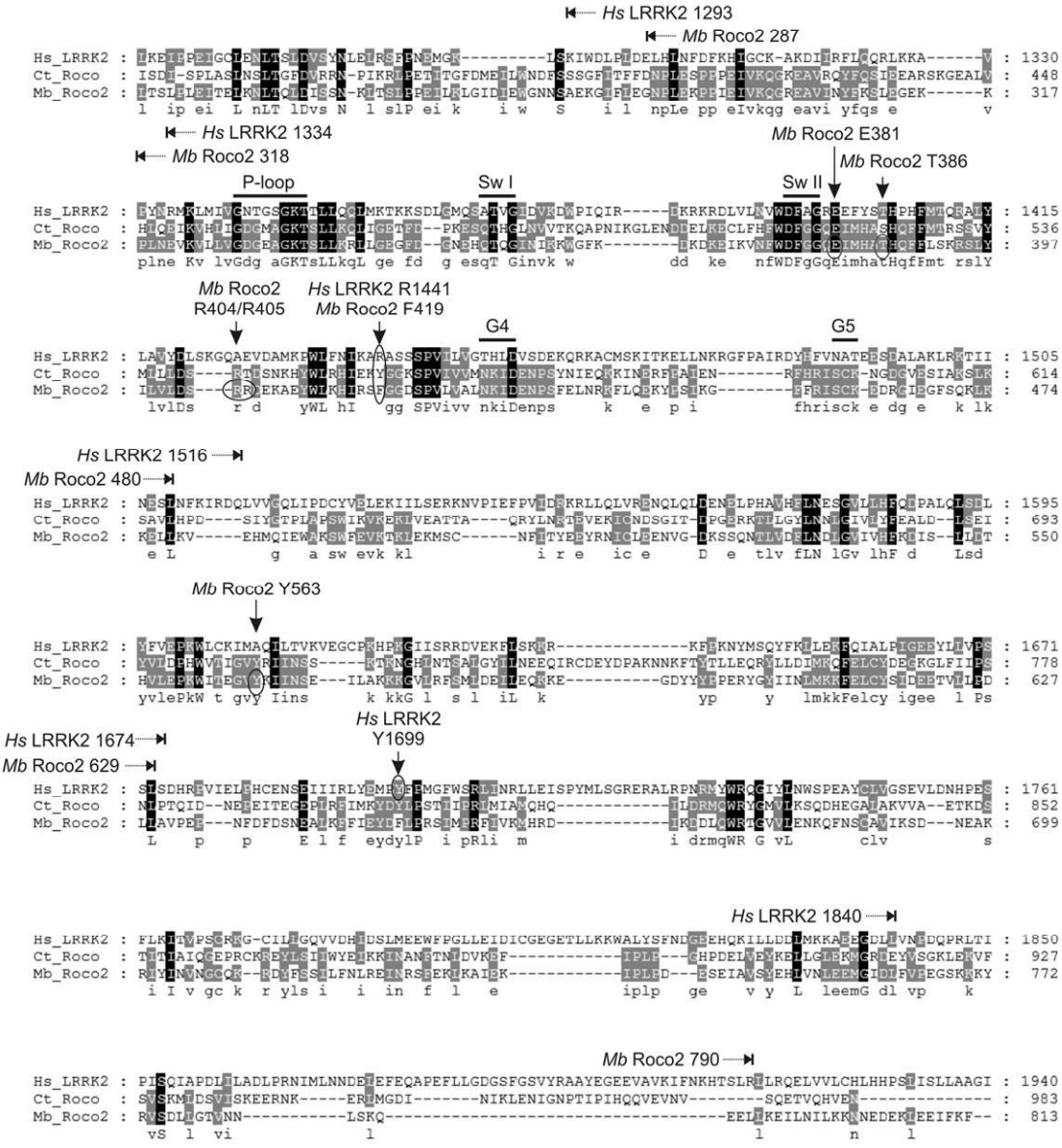

Figure S1. Sequence alignment. *Homo sapiens (Hs)* LRRK2 (Swiss: Q5S007), *Chlorobium tepidum* (*Ct*) Roco (Swiss: Q8KC98) and *Methanosarcina barkeri* (*Mb*) Roco2 (Swiss: Q46A62) sequences were aligned using the PRALINE sequence alignment server [1] and GeneDoc (http://www.psc.edu/biomed/genedoc). Black and grey background represents the sequence identity in three or two sequences, respectively. Structural elements of the G-domain (lines), construct boundaries (horizontal arrows), and important residues (vertical arrows) are indicated above the alignment.

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